

# TNF gene promoter region polymorphisms and association with young-onset rheumatoid arthritis

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**Abstract:** Rheumatoid arthritis (RA) is an inflammatory autoimmune disease that shares a major global economic burden due to disabilities and mortality risk. It affects all age groups with a female predominance. Tumor Necrosis Factor (TNF) a proinflammatory cytokine is one of the key players in etiology of autoimmune diseases such as RA. TNF gene promoter polymorphisms predict disease susceptibility, severity and therapeutic response. Therefore, the current case-control study was designed to evaluate the possible association of TNF gene promoter polymorphisms (-238 and -308) with susceptibility to young-onset RA. The study involves 102 individuals (50 young-onset RA patients, 52 healthy individuals). Genomic DNA was extracted using a standard phenol-chloroform method followed by PCR-RFLP for the screening of TNF gene promoter polymorphisms (-238 and -308). The study resulted in the association of TNF -238G/A polymorphism with susceptibility to young-onset RA in the homozygous form GG (Odds Ratio = 3.23, p-value= <0.05), though no significant difference was observed for -308G/A polymorphism among young-onset RA patients and controls. Thus concludes; TNF -238/G/A contributes to the risk of susceptibility to young-onset RA, conversely, TNF -308 G/A protects against the disease. Consequently, the study has demonstrated a possible relationship of studied TNF polymorphism with young-onset RA.

**Keywords:** Rheumatoid arthritis, tumor necrosis factor, autoimmune disease, Pakistan.

## INTRODUCTION

Rheumatoid arthritis (RA), a chronic disease with an autoimmune origin affects about 0.24% of the global population. The condition deteriorates the quality of life and socioeconomic status thus results in disabilities (Guo, 2018). Hence, contributes a burden of \$39.2 billion annually to the US only (Birnbaum, 2010). Although RA involves all age groups with female predominance, however; young-onset RA results in adverse forms with increased Rheumatoid Factor (RF) titer and high HAQ-DI (Disability Index) (El-Labban AS, 2010).

RA is characterized by loss of self-tolerance, presence of autoantibodies, and immune cell infiltration in the synovium, which triggers the release of proinflammatory cytokines in the synovial region of joints leading to chronic inflammation with erosive bone damage leading to bone deformities. TNF- $\alpha$ , a proinflammatory cytokine is a potential biomarker for susceptibility, progression, and treatment of RA. Once released, it either binds to TNFR1 (p55) or TNFR2 (p75) and activates a cascade of proinflammatory cytokines in synovium and results in osteoclast damage, neovascularization, decreased macrophage infiltration and synovial hyperplasia. Synovial hyperplasia leads to chronic inflammation and joint damage (Guo, 2018). Patients with RA have increased mRNA expression and higher serum TNF- $\alpha$  levels, associated with a marked level of autoantibodies (anti-CCP and RF) (Wei, 2015). TNF- $\alpha$  inhibition results

in suppression of inflammation and joint damage, hence effective in therapeutics of RA. However, 30% of the patients results in poor response to anti-TNF therapies (Cuppen, 2016). Variation in TNF expression and response to treatment (Tarnowski, 2016) provide evidence for functionally diverse TNF alleles that define susceptibility to RA (Li, 2018). Although association studies for RA and TNF gene polymorphisms have been extensively reported, however; it has been limited to adult-onset RA patients. Therefore, the current study aimed to investigate, an association of TNF gene promoter polymorphisms with young-onset RA patients in comparison with healthy individuals.

## MATERIALS AND METHODS

This case-control study was approved by ethical review board of The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, and Jinnah Postgraduate Medical Center (JPMC), Karachi, Pakistan. Fifty young-onset RA patients were recruited from Rheumatology clinic, JPMC after being confirmed for diagnosis of RA following 2010 ACR-EULAR criteria. Patients diagnosed with concomitant autoimmune disease and age above thirty years were excluded from the study. Fifty-two healthy individuals were considered as controls from the same population. All the participants were provided with informed consent for voluntary involvement in the study through verbal communication and written information sheet. Five ml of venous blood

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was collected from all participants in a sterile vacutainer containing Acid-Citrate-Dextrose (ACD). Genomic DNA was isolated using the Standard Phenol-Chloroform method. All isolated DNA samples were assessed for quality and quantity, using agarose gel electrophoresis and spectrophotometer respectively then dissolved in TE buffer (pH 8), diluted till 50ng and stored at -70°C till further process.

The site-specific amplification was performed by polymerase chain reaction and polymorphisms were investigated using RFLP. PCR was performed in a total volume of a 30µl reaction containing 1X KCL buffer, 5% DMSO, 1.4 mM MgCl<sub>2</sub>, 0.2µM primers, 200 µM dNTPs, 1U Taq DNA Polymerase (Thermo Scientific™) and 50 ng genomic-DNA. The amplification cycles were as: 94°C for 5 minutes, 35 cycles of denaturing at 94°C for 30 seconds, annealing at 59°C for 35 seconds, extension at 72°C for 30 seconds, and final extension for 10 minutes using Eppendorf Mastercycler Gradient 5331 thermocycler (Thermofisher). Amplified products were confirmed through 1.5% agarose gel electrophoresed visualized under UV light. These amplified products were digested by allele-specific restriction enzymes (Thermo Scientific™) overnight at 37°C. Resulted fragments were then analyzed with ultraviolet light after 3% agarose gel electrophoresed at 120V for 45min. The primers (Du, 2006) used for genotyping of -308G/A and -238G/A variation, size of the resulted PCR product, restriction enzyme and obtained fragments are listed in table 1.

**STATISTICAL ANALYSIS**

Pearson’s chi-square was performed to determine the level of statistical association between genotype and allele frequencies in RA patients and controls using StatCalc, Epi Info™ 7.2, Division of Health Informatics and Surveillance, Center for Surveillance, Epidemiology and Laboratory Services. Odds ratio and 95% Confidence Interval was determined using MedCalc Statistical Software for Windows, version 18.11, bvba, Ostend, Belgium.

**RESULTS**

The RA patients had a mean age of 21.92±5.07 years versus controls with mean age=18.5±5.6 years (mean±SD). In RA patients, females predominated the male (96% vs. 4%), whereas, duration of disease was 4.08±3.27 years. The seropositive patients prevailed to seronegative (68.6% vs. 31.4%). Analysis of -238G/A resulted in a higher frequency of homozygous GG genotype in RA patients vs. controls (90% vs. 69%), whereas, homozygous AA genotype was absent in RA patients vs. 3% in controls. [(OR=3.25; 95% CI=1.06-9.97; chi-square (χ<sup>2</sup>) =6.72(p<0.05); table 3)]. The heterozygous variant was higher in controls vs. patients controls vs (25% vs. 10%), (Odds ratio=0.333; 95% CI=0.11-1.02). Allele frequency distribution showed prevalence of G allele in RA patients than controls (90% vs. 82%); and shows an association with RA. [p-value <0.05; chi-square (χ<sup>2</sup>) 8.65 (table 3)]. Second polymorphism -308 G/A resulted in a higher frequency of homozygous GG in RA patients (90%) vs. (88%) controls. [OR=0.56; 95% CI=0.186-1.676], whereas homozygous AA variant was absent in controls vs. 8% in RA patients. (OR= 0.098; 95% CI=0.0052-1.877). Controls had a higher frequency of heterozygous GA (19%) vs. RA patients (4%). [Odds ratio= 5.85; 95% CI= 1.21-28.26; chi-square (χ<sup>2</sup>) =1.096]. Allele frequency distribution among both groups revealed that there is no significant difference among prevalence of both G and A alleles in controls and RA patients (table 2 and 3) and thus, not associated with the disease.

**DISCUSSION**

The results obtained from the present study suggest an association of -238G/A with susceptibility to young-onset RA. Homozygous genotype GG and allele G for -238G/A, a predictor of negative response to TNF inhibitors and severity of RA (Tarnowski, 2016), was prevalent in RA patients (p<0.05) whereas, higher frequency of heterozygous variant GA and homozygous AA observed

**Table 1:** Primer sequence and restriction enzymes for 238 and 308 TNF promoter polymorphism

SNP	Primer sequences (5’ to 3’)	Product	Restriction enzyme	Genotype
-238G/A	F-AGAAGACCCCCTCGGAACC	152bp	MspI	GG: 132 + 20, AA: 152 GA: 152 + 132 + 20
	R-ATCTGGAGGAAGCGGTAGTG			
-308G/A	F-AGGCAATAGGTTTTGAGGGCCATG	143bp	StyI	GG: 123 + 20, AA: 143 GA: 143 + 123 + 20
	R-ACACACAAGCATCAAGGATAC			

**Table 2:** Distribution of Genotype and allele among RA patients and controls for -238 and -308 G/A polymorphism

Genotype	-238G/A	RA patients	Controls	-308G/A	RA patients	Controls
	GG		45 (90%)	36 (69.23%)	GG	44 (88%)
GA		5 (10%)	13 (25%)	GA	2 (4%)	10 (19%)
AA		0 (0%)	3 (6.67%)	AA	4 (8%)	0
Allele	G	95 (95%)	85 (82%)	G	90 (90%)	94 (90%)
	A	5 (5%)	19 (18%)	A	10 (10%)	10 (10%)

**Table 3:** Odds ratio and 95% confidence interval (CI) and chi-square ( $\chi^2$ ) values for RA patients and controls

Genotype	OR	95% CI	chi-square ( $\chi^2$ )	Genotype	OR	95% CI	chi-square( $\chi^2$ )
-238 GG	3.23	1.06-9.97	p<0.05 (6.72)	-308 GG	0.56	0.186-1.676	p=0.295 (1.096)
-238 GA	0.333	0.11-1.02		-308 GA	5.85	1.21-28.26	
-238 AA	0.14	0.007-2.78		-308 AA	0.098	0.0052-1.877	
Allele				Allele			
G	4.25	1.52-11.87	p=0.295 (1.096)	G	1.478	0.54-4.05	p=0.44 (0.579)
A	0.23	0.08-0.66		A	0.677	0.25-1.86	

in controls thus, may be protective against the disease. The absence of A allele in RA patients suggests its protective role against the disease. Our findings are consistent with the previous reports such as -238G/A polymorphism at promoter region is a risk factor for RA in Turkish, Macedonians, Han Chinese and Mexican population whereas contradictory to no association for Caucasians and Iranian patients (EL-Tahan, 2016; Hadinedoushan, 2016; Song, 2014). Investigation of -308G/A resulted in a higher frequency of genotype GG (90% vs. 88%) in RA patients whereas heterozygous GA was prevalent in control group (19% vs. 4%). Hence predicts -308 GG to be associated with RA as contrary to GA being protective against the disease. The absence of homozygous AA in controls (0% vs. 6%), may suggests its relevance with the disease. However, these differences in genotypic distribution were not statistically significant ( $p>0.05$ ) for association with RA in this study (table 2, 3). Our results are consistent with the previous reports as a meta-analysis of 19 studies reveals no association between RA and TNF -308 A allele. Conversely, no association was observed for homozygous AA in patients of Arab, European, and Asian origin and significantly associated with patients from Latin America (Song, 2014). Homozygous A allele is a risk factor for severity of RA in Brazilian patients, Czech and Han Chinese population whereas G allele is associated with severity of RA in Saudi populations (Al-Rayes, 2011; El-Tahan, 2016; Song, 2014). The contradiction among previous studies and current study may be attributed to ethnic variations in the TNF gene polymorphisms and by its relatedness MHC. The current study is an initiative to explore the association of TNF gene with young-onset RA patients. In future, further analysis is required on large scale, to investigate the association of TNF gene variations and response to anti-TNF therapies. This may be helpful for the development of personalized therapy for RA patients based on genotype and may result in progressive treatment response at earlier stage of disease.

## CONCLUSION

In conclusion, the association of TNF gene promoter polymorphisms with young-onset RA has not established earlier from the studied population. This study reveals an association with -238G/A polymorphism and conversely suggests no association with -308G/A polymorphism. In future, this initiative may help in prediction of effective response and treatment strategies for RA at early stage.

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